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The Aryl hydrocarbon receptor links Th17-mediated autoimmunity to environmental toxins

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Abstract

The aryl hydrocarbon receptor (AhR) is a ligand dependent transcription factor best known for mediating the toxicity of dioxin[1]. Environmental factors are believed to contribute to the increased prevalence of autoimmune diseases, many of which are due to the activity of T_H17 T cells. We show here that in the CD4 T cell lineage of mice AhR expression is restricted to the T_H17 subset and that its ligation results in the production of the T_H17 cytokine IL-22. AhR is also expressed in human Th17 cells. Activation of AhR by a high affinity ligand during T_H17 development markedly increases the proportion of T_H17 T cells and their production of cytokines. CD4 T cells from AhR deficient mice can develop T_H17 responses, but when confronted with AhR ligand fail to produce IL-22 and do not show enhanced T_H17 development. AhR activation during induction of experimental autoimmune encephalomyelitis (EAE) causes accelerated onset and increased pathology in wildtype, but not AhR deficient mice. AhR ligands may therefore represent co-factors in the development of autoimmune diseases.

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates a range of critical cellular events in response to halogenated aromatic hydrocarbons and non-halogenated polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)[1]. AhR expression is ubiquitous in vertebrate cells suggesting important and widespread roles, but the physiological role of AhR is not understood yet [2]. Mice with a targeted mutation of the AhR gene provided unequivocal evidence that the AhR is crucial to TCDD-induced toxicity and suggested a function for AhR in liver development [3-5].

Gene array analysis of CD4 effector T cell subsets showed that the T_H17 CD4 T cell subset, in addition to the lineage defining transcription factor RORγt[6], expresses AhR. Quantitative PCR analysis of CD4 effector T cell subsets from wildtype and AhR deficient B6 mice established that the lineage defining transcription factors Tbet, GATA-3, and RORγt respectively are expressed in a comparable manner as are the marker cytokines IFNγ, IL-4, IL-17A and IL-17F (Fig.1**a).** There is also similar Foxp3 expression in CD4 T cells from B6 and AhR deficient mice (supplementary Fig.1**b**). AhR was only induced under T_H17 conditions (IL-6 and TGFβ) with levels of expression similar to liver (Fig.1**a**,**d).** Under our conditions we found no AhR expression in cultures with TGFβ alone (iTreg) or IL-6 alone and no expression in natural Treg (supplementary Fig.1**a**). Importantly, AhR expression is also found in human T_H17 cells (Fig.1**b**).

While CD4 T cells cells from AhR deficient mice could differentiate into T_H17 cells, they lacked, however, the expression of IL-22 (Fig.1**c**). IL-22, originally defined as an hepatocyte stimulating factor[7] is co-expressed with IL-17 by T_H17 T cells and its

expression is thought to be enhanced by dendritic cell-derived IL-23 [8, 9]. The biological functions of IL-22 are not fully understood; on the one hand IL-22 appears to be pro-inflammatory, inducing dermal inflammation and proinflammatory gene expression in the skin [8, 10], on the other hand IL-22 delivery ameliorates T cell mediated liver injury in T cell mediated hepatitis [11, 12].

AhR resides in the cytoplasm complexed with hsp90 until binding of ligand triggers conformational changes resulting in an exchange of hsp90 for the nuclear translocation component ARNT, reviewed in [1]. ARNT was found expressed in all CD4 T cell subsets (data not shown). Ligation of AhR by 6-formylindolo[3,2-b] carbazole (FICZ), a tryptophan derived photoproduct that is thought to be an endogenous ligand with high affinity for the AhR receptor upregulates genes encoding xenobiotic metabolizing cytochrome P450 enzymes such as CYP1A1 [13].

In order to test whether exposure of T cells to FICZ influences differentiation of naïve CD4 T cells to effector cells, we added FICZ during the in vitro differentiation of CD4 effector T cell subsets. The addition of FICZ did not induce AhR or its downstream target CYP1A1 in T_H0, T_H1, T_H2 and iTreg T cell subsets (Fig.2**a**) and did not alter their expression of IFNγ, IL-4, IL-21 nor their lineage defining transcription factors (supplementary Fig.1**c**). However, the presence of FICZ during T_H17 inducing conditions led to strong upregulation of IL-17A, IL-17 F and particularly of IL-22 mRNA expression (Fig.2**a** and supplementary Fig.2**a**).

Comparison of Th17 differentiation in CD4 T cells from wildtype B6 and AhR deficient mice by intracellular staining showed that exposure of B6 CD4 T cells to FICZ under T_H17 conditions strongly enhanced IL-17A/F production and increased the proportion

and staining intensity of cells producing IL-22 (Fig.2**b** top panels**,** supplementary Fig.2**b**). In contrast, IL-17A/F production was attenuated in T_H17 from AhR deficient mice and no IL-22 was detectable whether FICZ was present or not (Fig.2**b** bottom panels). A similar response was seen with another AhR ligand, β–Naphthoflavone, which has lower affinity for AhR and consequently requires about a 10 fold higher dose compared with FICZ (supplementary Fig.2**c**). Similar to mouse also human Th17 cells reacted to AhR ligation with increased expression of IL-17A, IL-17F and IL-22 as well as induction of CYP1A1 (Fig.2**c**).

In order to test whether AhR expression on its own is essential and sufficient to drive IL-22 expression, we performed retroviral transduction of sorted naïve AhR deficient CD4 T cells with an AhR-GFP construct or a GFP vector control construct. Transduction under neutral, Th1, Th2 or iTreg conditions did not reconstitute IL-22 expression even in the presence of FICZ (Supplementary Fig.3). However, under T_H17 conditions, reconstitution of AhR expression by retroviral transduction induced expression of IL-22 (Fig.3**a** top right panel) and increased the proportion of IL-17 producing cells (Fig.3**b** top right panel). Exposure to FICZ resulted in a substantial increase in IL-22 (Fig.3**a** bottom right panel) as well as the enhanced expression of IL-17A (Fig.3**b** bottom right panel). RT-PCR analysis of cultured AhR transduced Th17 cells confirmed the increase in IL-17 expression, the enhancement of IL-22 as well as the induction of the AhR target CYP1A1 upon exposure to FICZ (Fig.3**c**). Thus, AhR expression is only functional in CD4 T cells that have differentiated to the T_H17 lineage.

Next we immunized B6 mice with MOG peptide in complete Freunds adjuvant (CFA), removed draining lymph nodes 7 days later and isolated $CCR6^+$ CD4 T cells which are enriched in T_H17 cells[14, 15] to test whether AhR is also expressed in Th17 cells generated in vivo. $CCR6^+$ CD4 T cells expressed AhR, IL-17A, F, IL-22 and RORγt, whereas the CCR6 negative CD4 T cell fraction lacked expression of AhR and T_H17 markers, confirming that physiological differentiation in vivo recapitulates in vitro differentiation (Fig.4**a**).

T_H17 T cells play a prominent role in the pathology of autoimmune diseases such as EAE, which is induced by immunization with MOG peptide 35-55 and CFA. Analysis of spinal cord at the height of the EAE response on day 18 showed increased numbers of CD4 T cells producing IL-17 and IL-22 in B6 mice treated with MOG/CFA and FICZ, whereas AhR deficient mice showed reduced numbers of IL-17 producing cells and minimal numbers of IL-22 producers (Fig.4**b**). There was no difference in the numbers of Foxp3 expressing regulatory T cells in spinal cord in the three groups of mice.

While B6 mice developed EAE with a mean day of onset of 13.9, AhR deficient mice developed EAE with delayed kinetics (mean day of onset 15.6) in line with the attenuated T_H17 differentiation seen in vitro. Despite the delayed onset of EAE, most AhR deficient mice succumbed to disease eventually (Fig.4**c**,**e**). Stimulation of AhR by inclusion of FICZ in the antigen emulsion accelerated the onset (day 11.7) and increased the severity of EAE in B6 mice, but, as expected, did not influence the onset or severity of EAE in AhR deficient mice (day 15.8) (Fig.4**c,e**). To assess the influence of AhR deficiency in haematopoietic or non-haematopoietic cells, we also induced EAE in chimeras

constructed either by injection of AhR deficient bone marrow into irradiated wild type mice (AhR-B6) or by injection of B6 wild type bone marrow into irradiated AhR deficient mice (B6-AhR). AhR-B6 chimeras showed attenuated EAE like AhR deficient mice, whereas B6-AhR chimeras developed EAE with kinetics and severity similar to wild type mice (Fig.4**d**).

Studies on the effect of AhR stimulation on the immune system have so far focused exclusively on TCDD as a ligand because of its toxicological relevance. Although adverse effects of TCDD on immune responses are well documented[16], no direct measurements of AhR expression on highly purified polarized subsets of CD4 T cells have been reported. TCDD, which cannot be metabolized and therefore causes prolonged stimulation of AhR in many cells of the body, is known to induce profound suppression of immune responses in wild type, but not AhR deficient mice [16], but despite decades of research the underlying mechanisms for this profound toxicity remain unclear. More recently it has been suggested that TCDD promotes the generation of regulatory T cells [17], while causing the premature decline of activated CD4 T cells. In our hands, stimulation of AhR by FICZ did not influence the number of regulatory T cells during MOG induced EAE responses (see Fig.4b). It is conceivable that the high toxicity of TCDD for effector cells causes a proportional shift in regulatory T cells which are in general more resistant to many depletion regimes[18, 19], due to the death of other cells, rather than an actual expansion in numbers. While TCDD may be the classical AhR ligand used in the toxicology field to analyse the effects of AhR activation, it is clear that it is not a natural ligand and it is widely recognized that pollutants such as TCDD are

unlikely to have provided the evolutionary pressure for the function of this highly conserved system. There is ongoing debate in the toxicology field about what is the most relevant physiological ligand for AhR [20]. Nevertheless, compelling indirect evidence shows that UV photoproducts of tryptophan such as the high affinity ligand FICZ studied in our paper, could be synthesized in vivo as exposure of human skin to UV induces CYP1A1[21]. Future studies focusing on additional physiological ligands of AhR which can be metabolized by the CYP1 enzymes and therefore cause only transient AhR activation, may give further insights into the consequences of AhR stimulation in T_H17 cells.

Our data here show that the AhR, in addition to promoting the expression of IL-22, enhances T_H17 development and the expression levels of IL-17A/F and consequently increases autoimmune pathology. Blockade of IL-17A with an auto-vaccine as well as neutralizing antibody can completely prevent the development of EAE [22 23] emphasizing the central role of IL-17A in the pathogenesis of this autoimmune disease. Although it has been suggested that IL-22 may contribute to autoimmune pathology since, like IL-17, it disrupts blood-brain barrier tight junctions[24], IL-22 deficient mice do not seem to have altered susceptibility to EAE induction[25], suggesting that this cytokine may be dispensable for the development of pathology. Thus, the enhancement of IL-17 production by AhR ligation may be more crucial than the induction of IL-22 in determining disease severity.

It is currently thought that IL-22 expression depends on induction by IL-23[8, 9]. Restimulation of lymph node cells from mice immunized with MOG/CFA or MOG/CFA and FICZ in the presence or absence of IL-23 showed that IL-23 increased the proportion

of IL-17 producing cells both from wild type and AhR deficient mice (supplementary Fig.4left), but only had a small effect on production of IL-22 (supplementary Fig.4b right), suggesting that IL-23 has some effect independent of AhR ligation, although clearly stimulation of AhR appears to be the dominant pathway for IL-22 production.

How AhR interacts with the T_H17 pathway is currently unknown, but there is a substantial amount of literature (reviewed in [26]) describing interactions of AhR with other key regulatory proteins including for instance NF-kB[27] , which plays a role in the induction of EAE[28]. The core nucleotide sequence to which the nuclear AHR complex binds, also termed the 'xenobiotic responsive element' [29] occurs frequently in the mammalian genome and is also represented in IL-17A,F, IL-22 and RORγt. While basal expression of AhR and IL-22 is detectable in T_H17 T cells in the apparent absence of a ligand, there are numerous endogenous agents that can activate AhR, such as prostaglandins, bilirubin at high concentration, modified low-density lipoprotein (LDL) and various modifications of tryptophan whose UV irradiated photoconversion into the high affinity ligand FICZ is only one example (reviewed in [20]).

Autoimmune diseases are multifactorial, depending on intrinsic components such as genetics, hormones or age and environmental factors, including infections, diet, drugs and chemicals. The increasing prevalence of certain autoimmune diseases in highly industrialized countries is likely to be connected to such environmental factors.

Our data linking a transcription factor responsive to environmental pollutants to the T_H17 program opens intriguing possibilities regarding the potential of such factors to initiate or

augment autoimmune conditions and warrant closer examination of a possible role of AhR in human autoimmune diseases.

Methods:

Mice. C57Bl/6 (B6) and AhR deficient mice on a B6 background (B6 BRA AhRKO) [3] originally obtained from the Jackson Laboratory via Dr. A. Smith at Leicester University were bred in the SPF facility at NIMR. All animal experiments were done according to institutional guidelines and Home Office regulations.

Human T cell culture:

Human PBMC from a healthy volunteer were isolated by Ficoll/Paque, CD4 T cells were purified by magnetic sorting and cultured at $1.5x10^5$/cells per well in plates coated with anti-CD3 (1μg/ml) and anti-CD28 (1μg/ml) in the presence of 10ng/ml IL-1 and 40ng/ml IL-6 (T_H17 condition) or 3ng/ml IL-12, 10ng/ml IL-2 and 10μg/ml anti-TGFβ (T_H1 condition). qPCR analysis for T_H17 markers and AhR expression was performed on day 4.

In vitro T cell differentiation and cytokine staining

Detailed description of procedures is given in supplementary information.

Real time PCR.

The expression of mRNA for transcription factors and cytokines in CD4 T cell subsets was analysed 4-5 days after T cell activation using specific primers from Applied Biosystems and expression was normalized to the housekeeping gene HPRT. More details and a list of primers used can be found in Supplementary Methods.

Retroviral transduction. AhR was cloned into vector pIRES2-EGFP (Clontech) generating a bicistronic mRNA encoding AhR and, separated by an IRES element, EGFP. Viruses were generated by simultaneous $CaCl_2$-mediated transient transfections of 293T cells with three plasmids providing vector, gag-pol, and env functions. Details of the transduction protocol are given in supplementary Methods.

EAE induction. EAE was induced and scored as described previously[30]. Some mice received 600ng FICZ in the antigen emulsion. Draining lymph nodes were isolated 7 days after immunization. Spinal cord was isolated on day 18 after EAE induction for determination of cell numbers.

Legends

Fig.1 AhR is selectively expressed in the Th17 subset

a) FACS sorted naïve CD4 T cells from B6 or AhR deficient mice stimulated under T_H0, T_H2, iTreg or T_H17 conditions and harvested on day 5 for qPCR. mRNA levels, normalised to Hprt expression are shown. **b)** mRNA levels of AhR in human CD4 T cells stimulated under Th1 or Th17 conditions **c**: mRNA levels for IL-22 in CD4 T cells subsets from B6 or AhR deficient mice **d**: mRNA levels of AhR in CD4 T cell subsets and T_H17 cells from three mice compared with mouse liver.

Fig.2 AhR ligation promotes the Th17 program

a) Mean mRNA levels +/- s.d. from B6 CD4 effector subsets generated in the presence or absence of 200nM FICZ. **b,c)** CD4 T cells from B6 (top panels) and AhR deficient mice (lower panels) cultured under Th17 conditions in the presence or absence of 200nM FICZ and stained for expression of IL-17A vs IL-17F (**b**) and IL-17A vs IL-22 (**c**) after restimulation with PdBU/ionomycin. A summary of all experiments is shown in supplementary Fig. 2**b**. **c)** mRNA levels of IL-17A, IL-17F, IL-22 and CYP1A1 in human CD4 T cells stimulated under Th17 conditions in the presence or absence of 3μM β−NF.

Fig.3 Retroviral transduction of AhR restores IL-22 expression

a,b) FACS sorted naïve CD4 T cells from AhR deficient mice cultured under T_H17 conditions and transduced with vector control (RV-GFP) or AhR containing construct

(RV-AhR –GFP) in the presence (lower panels) or absence (upper panels) of FICZ. IL-22 intracellular staining vs GFP expression **a**) and IL-17A expression in gated GFP^+ cells (**b**) were assessed. **c)** qPCR for IL-17A, IL-22 and CYP1A1 in T_H17 cells from AhR deficient mice transduced with control retroviral vector, control vector in the presence of 200nM FICZ AhR containing vector or AhR containing vector in the presence of FICZ.

Fig.4. EAE is enhanced by AhR ligation

a) RT-PCR analysis for IL-17A, F, IL-22, RORγt and AhR of FACS sorted $CCR6^-$ CD4 T cells or $CCR6^+$ CD4 T cells from draining lymph nodes 7 days after MOG immunization.

b) mean numbers of IL-17A, IL-22 and FoxP3 cells in spinal cord 18 days after immunization of B6 mice (n=4) with MOG or MOG+ FICZ and MOG immunized AhR deficient mice n=4. **c**) Mean clinical scores +/- SEM of B6 (n=12) or AhR deficient mice (n= 12) immunized with MOG in the absence or presence of 600ng FICZ. **d**) Mean clinical scores+/- SEM of MOG immunized chimeras from AhR deficient donors into irradiated B6 hosts (AhR-B6 n=8), or bone marrow from B6 donors into irradiated AhR deficient hosts (B6-Ahr n=8). **e**) incidence, mean day of onset and mean maximal scores for the mice in **c**, **d**. p values are p= 0.0013 for mean day of onset in B6 vs B6+FICZ and p= 0.03 for B6 vs AhR.

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M. Veldhoen and K.Hirota performed the experiments, A.Westendorf and J.Buer did the microarrays and analysis, L.Dumoutier and J-C.Renauld generated and provided the anti-IL-22 antibody, B. Stockinger* directed the research and wrote the manuscript.

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